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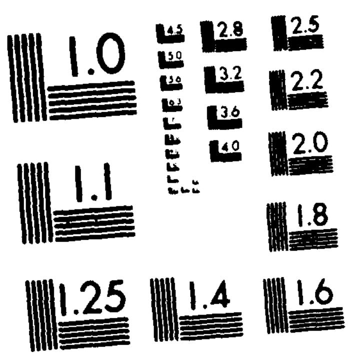
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A commercially available system was used to determine the relative response of bioluminescent bacteria to a number of alkyltin compounds: R_4Sn, R_3SnX, R_2SnX_2, and $RSnX_3$, where R = alkyl group and X = halide. Within a series of compounds differing only in the number of R groups attached to the central tin atom, the most toxic compound was always the trialkyltin compound. The greatest difference in toxicity was found in the butyltin series of compounds; tributyltin was ≈35 times more toxic than dibutyltin and ≈750 times more toxic than (mono)butyltin. When trialkyltin compounds were compared, the toxicity to these bacteria increased with the number of carbons in the alkyl chain; the tributyltin compounds are ≈150 times more toxic than trimethyltin compounds.

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RESPONSE OF BIOLUMINESCENT BACTERIA TO ALKYLTIN COMPOUNDS.

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ABSTRACT

The reduction of light intensity in bioluminescent bacteria upon exposure to toxic substances can be used for rapid screening of materials. Results are often comparable to more expensive standard bioassays.

A commercially available system was used to determine the relative response of bioluminescent bacteria to a number of alkyltin compounds: R_4Sn, R_3SnX, R_2SnX_2, and $RSnX_3$, where R = alkyl group and X = halide. Within a series of compounds differing only in the number of R groups attached to the central tin atom, the most toxic compound was always the trialkyltin compound. The greatest difference in toxicity was found in the butyltin series of compounds; tributyltin was ~35 times more toxic than dibutyltin and ~750 times more toxic than (mono)butyltin. When trialkyltin compounds were compared, the toxicity to these bacteria increased with the number of carbons in the alkyl chain; the tributyltin compounds are ~150 times more toxic than trimethyltin compounds.

INTRODUCTION

The biocidal effects of alkyltin compounds at part per billion levels make these compounds ideal candidates for such uses as antifouling ship paints, fungicides and wood preservatives. This paper compares the relative toxicities of a number of alkyltin compounds using a highly reproducible, simple and rapid bioassay using bioluminescent bacteria (Microtox[R]). To our knowledge there is no published information on the relative toxicity of the alkyltin compounds to these microbes.

The Microtox[R] system can produce a bioassay within 30 minutes and has been shown to have similar sensitivity and generally a good correlation to more time consuming and difficult bioassays. DeZwart and Slooff (1) demonstrated that Microtox[R] sensitivity compared favorably with 20 other aquatic bioassays. Lebsack et al.(2) tested fossil fuel process water and pure phenolic compounds, which are constituents of this water, and found that Microtox[R] results were comparable to both static and flow-through acute fish bioassays. Qureshi et al. (3) found , in most cases, that Microtox[R] exhibited similar levels of sensitivity as did fish, crustacea, and other bacterial bioassays, particularly toward organic compounds and complex effluents. Likewise, Bulich et al. (4) showed good correlations between Microtox[R] and fish bioassays for pure compounds and complex effluents. The Microtox[R] bioassay is a convenient way to compare the relative toxicity of compounds, and to demonstrate degradation of compounds as a decrease in toxicity (5) and a method to study synergistic and antagonistic effects of compounds (6).

Ribo and Kaiser (7) used Microtox[R] to calculate structure-activity correlations with physico-chemical parameters. A good correlation existed between Microtox[R] toxicity and the molar refractivity parameters of para-substituted phenols and also the octanol/water partition coefficients of chloro-substitutents in the benzene ring of chlorobenzenes. Curtis et al. (8) demonstrated a high correlation between Microtox[R] toxicity and fish bioassays in semihomologous series of alcohols, ketones and ethane derivatives. Toxicity for Microtox[R] and fish increased as the number of carbons increased for

each semihomologous series.

METHODS AND MATERIALS

Tri-n-butyltin chloride, tri-n-butyltin bromide, triethyltin bromide, trimethyltin chloride, dimethyltin dichloride, methyltin trichloride, diethyltin dichloride, di-n-butyltin dichloride, tetrabutyltin and tetraallyltin were obtained from Alfa Products (Danvers, MA). Di-n-propyltin dichloride, tri-n-propyltin chloride, n-butyltin trichloride and triphenyltin chloride were obtained from Pfaltz and Bauer, Inc. (Waterbury, CT). The chemicals were used without further purification. Tetrapentyltin was prepared by reaction of $SnCl_4$ (Alfa Products, Danvers, MA) with n-pentylmagnesium bromide (Alfa Products, Danvers, MA). Tetrabut-1-enyltin, tetrabut-2-enyltin, and tetrabut-3-enyltin were prepared by reaction of $SnCl_4$ with the appropriate freshly prepared Grignard Reagent. Tri-n-pentyltin bromide, tribut-1-enyltin bromide, tribut-3-enyltin bromide and dibut-3-enyltin dibromide were prepared by bromination of the symmetrical tetraorganotin compound. These compounds were purified by vacuum distillation and column chromatography on Florisil (Supelco, Inc., Bellefonte, PA).

For Microtox^R testing, stock solutions of the compounds were prepared in 95% ethanol at ~1-2 mg/ml. Appropriate amounts of the ethanol solutions were added to 2% aqueous NaCl to achieve a workable concentration while keeping the ethanol concentration as low as possible. Typically the ethanol concentration was about 0.05%. Where higher concentrations of ethanol were required owing to solubility problems with the stock solution, an ethanol blank was also prepared for the Microtox^R control.

The Microtox^R Toxicity Analyzer Model 2055, manufactured by Microbics Corporation, Carlsbad, CA, is basically a photometer with variable temperature control (9,10). The bioassay measures the relative reduction in light output by a luminescent bacterium, *Photobacterium phosphoreum* NRRL B-11177 when exposed to a toxicant. The light output is a normal metabolic function of these bacteria once rehydrated with distilled water. The bacteria are provided in a convenient freeze-dried form by Microbics Corporation and are immediately activated by the addition of 1 ml distilled water. Upon rehydration, the bacterial suspension is used as a reagent by adding 10 ul to each sample. Since the bacteria are marine, 2% NaCl is used to provide osmotic protection to the bacterial cells.

Serial dilutions of each compound for measurement are performed in the Microtox^R photometer/incubator at 15 °C. Controls consist of triplicate 1 ml portions of 2% NaCl and candidate toxics are prepared in and subsequently serially diluted in 2% NaCl, with a final volume of 1 ml for each dilution. After a 5 minute period for temperature equilibration, 10 ul of reagent (rehydrated bacteria) is added to each of the controls and the serial dilutions of the test compound. Measurements in the photometer are made at 5 and 15 minutes after addition of the reagent. This procedure is repeated at least four separate times for each compound to provide four independent toxicity values.

The toxicity value is expressed as an EC50 concentration, which is the concentration of a compound which caused a 50% reduction in light output. The EC50 concentrations were determined by graphic interpolation on log-log paper, plotting the gamma function against concentration. The gamma function is the ratio of the amount of light lost to the amount of light remaining. A gamma value of 1 corresponds to a 50% reduction in light, or EC50. The EC50 values at 5 and 15 minutes are reported here since these times are most commonly reported in the literature when Microtox^R is compared to fish and other bioassays. A low EC50 value indicates high toxicity.

RESULTS

The EC50 values at 5 and 15 minutes for the methyl-, ethyl-, propyl- and butyltins are shown in Figures 1,2,3, and 4, respectively, as a function of the number of alkyl groups (R) attached to the tin atom. In each case, the EC50 decreases and, hence toxicity increases, from (mono)alkyltin to trialkyltin. Toxicity decreases from trialkyltin to tetraalkyltin compounds. The largest increase in toxicity was found for the butyltin compounds, where tributyltin was found to be ~750 times more toxic than the monobutyltin. The greatest difference in toxicity was also found between dibutyl- and tributyltin compounds among the series tested. The dialkyl- to trialkyltin difference in toxicity increased as the number of carbons in the R groups increased.

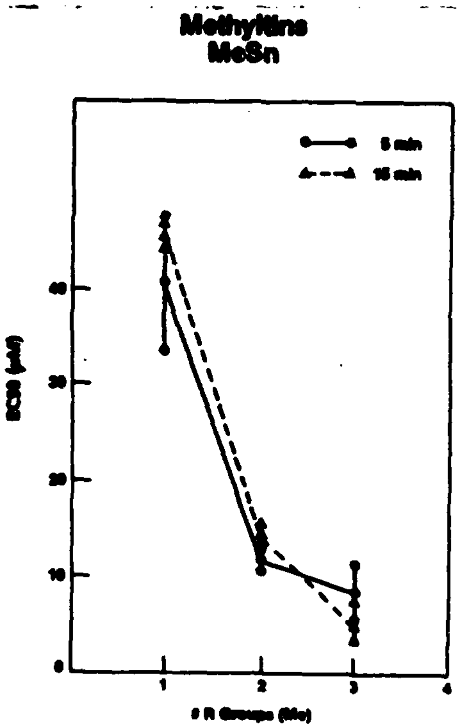

Figure 1. EC50 values for methyltin trichloride, dimethyltin dichloride and trimethyltin chloride.

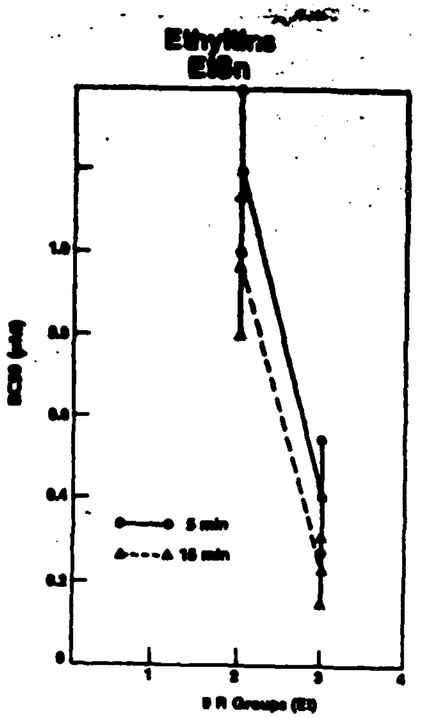

Figure 2. EC50 values for diethyltin dichloride and triethyltin bromide.

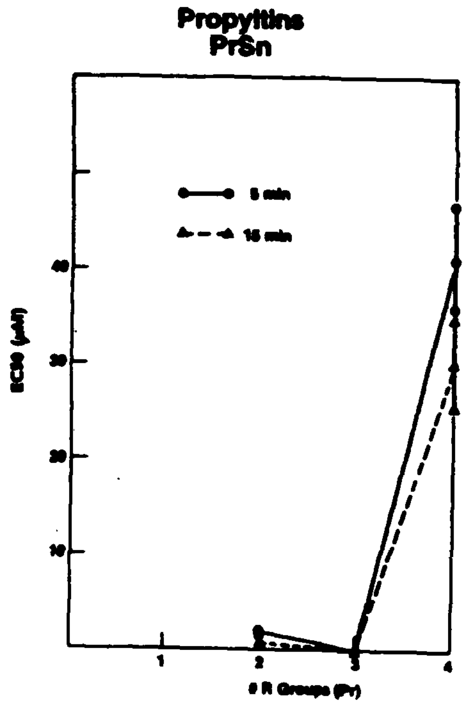

Figure 3. EC50 vlaues for dipropyltin dichloride, tripropyltin chloride and tetrapropyltin.

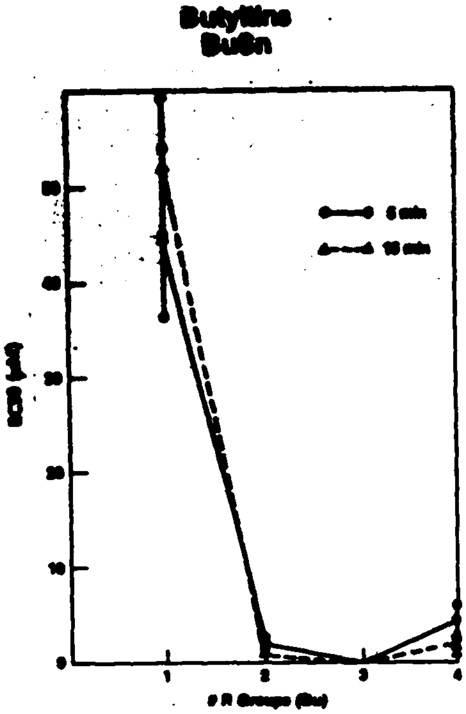

Figure 4. EC50 values for butyltin trichloride, dibutyltin dichloride tributyltin chloride and tetrabutyltin.

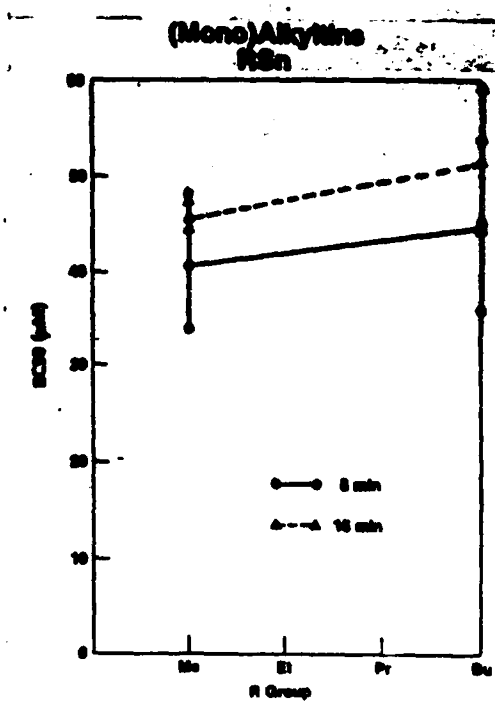

Figure 5. EC50 values for (mono)alkyltin trihalides.

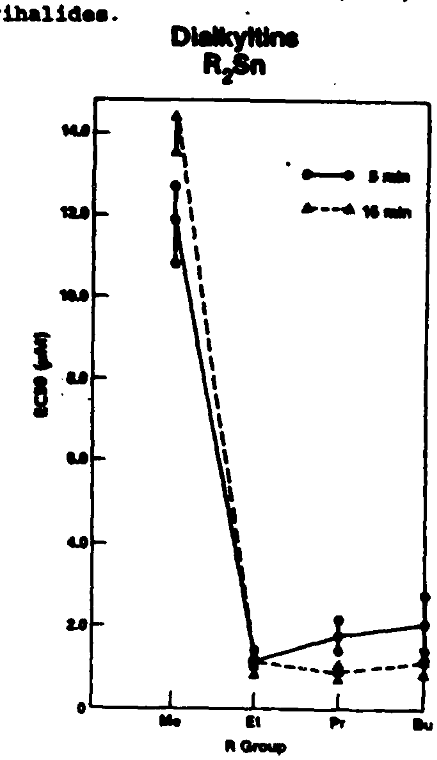

Figure 6. EC50 values for dialkyltin dihalides.

Figures 5, 6, 7 and 8 show the EC50 at 5 and 15 minutes for mono-, di-, tri- and tetraalkyltins, respectively, as a function of the number of carbons in the alkyl chain. As seen in Figure 5, the toxicity of methyl and butyltin is approximately the same. For the dialkyltin compounds, shown in Figure 6, toxicity increased dramatically from dimethyl- to diethyltin and was approximately the same for diethyl-, dipropyl- and dibutyltin. The greatest range in toxicity is shown in Figure 7 for the trialkyltin compounds, where tributyltin was found to be ~150 times more toxic than trimethyltin. Toxicity decreased again from tributyltin to tripentyltin. The greatest toxicity was found for butyltin species in the tetraalkyltin compounds, shown in Figure 8, as well. The EC50 for tetrapentyltin could only be determined as a "greater than value" owing to solubility problems.

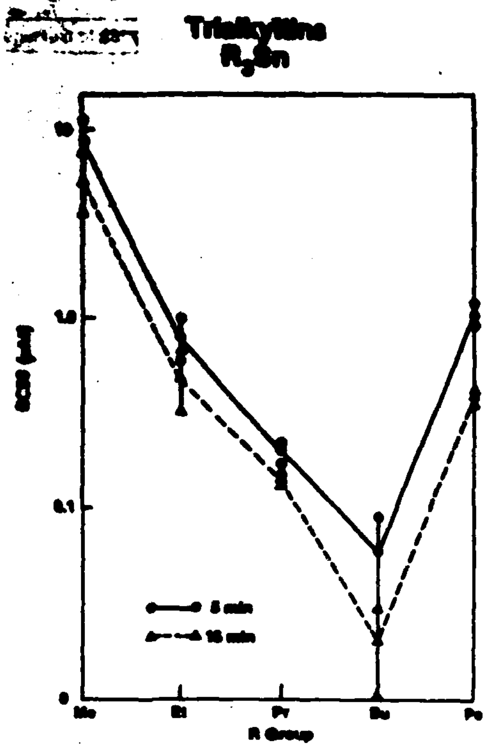

Figure 7. EC50 values for trialkyltin halides.

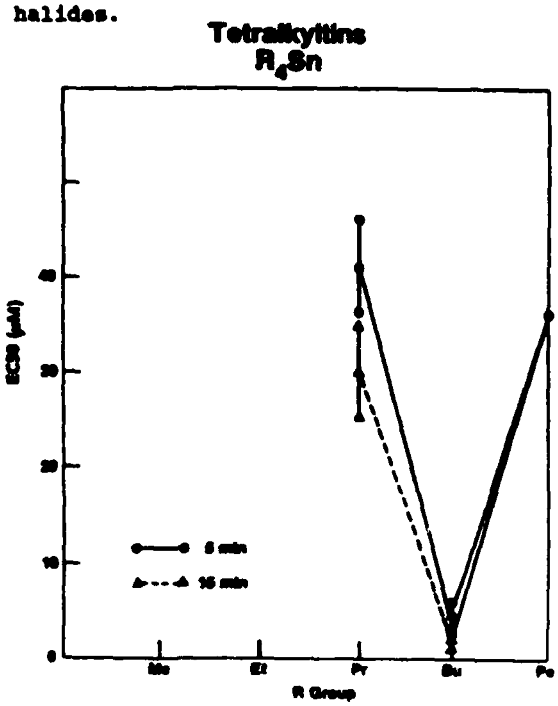

Figure 8. EC50 values for tetraalkyltins.

Trialkyltin compounds show considerable variation in species response (11,12) Maximum toxicity in a homologous series of compounds differing only in carbon chain length was shown to be trimethyltin for insects, triethyltin for mammals, and tripropyltin for gram-negative bacteria. Gram-positive bacteria, fish and fungi all show maximum response to tributyltins. The bioluminescent bacteria, Photobacterium phosphoreum, although gram-negative, showed maximum sensitivity to tributyltin compounds. The quantitative results are very similar to LC50 data measured by primary productivity in freshwater diatoms (13) and to LC50 determined by viable cell counts in gram-negative bacteria associated with fecal pollution (14). As also reported by Sijesteinjn et al. (12), the toxicity decreased with increasing carbon chain length beyond the point of maximum toxicity.

Bioaccumulation and biochemical activity have been shown to be dependent on octanol/water partition coefficients (15,16,17). Although data on water-octanol partition coefficients and on correct values for the metal-substituent bonds to calculate the Hanch pi parameters are not available Laughlin et al.(18) showed correlation between toxicity to mud crab larvae and the Hansch pi parameter for the carbon chain fragment. Using bioluminescent bacteria, we could show correlation only for the trialkyltins from methyl through butyl only. Pentyltin compounds, triphenyltin and the experimental compounds mentioned below showed anomalous behavior.

The Microtox[R] system was also used to determine the toxicities of some experimental compounds. Table 1 shows the EC50 for some symmetrical unsaturated tetraorganotin compounds and their saturated analogues. Tetraallyltin shows greater toxicity than tetrapropyltin. Tetrabutenyltins with double bonds in the 1, 2 and 3 positions exhibit EC50s different from each other and generally lower than tetrabutyltin. These results are contrary to expectation, since the polarity induced by the double bond should cause a lower

partition coefficient and result in a lower toxicity (17). Diallyldibutyltin exhibits an EC50 intermediate between tetraallyltin and tetrabutyltin, as might be expected.

TABLE 1

COMPARISON OF SATURATED AND UNSATURATED TETRAORGANOTINS

Compound	5 min EC50 (uM)	15 min EC50 (uM)
Pr_4Sn	41.10 + 5.14	30.14 + 4.79
All_4Sn	19.37 + 3.38	14.79 + 3.87
All_2Bu_2Sn	6.65 + 1.14	4.11 + 1.20
Bu_4Sn	4.31 + 1.70	2.10 + 0.95
$Bu_4(3)Sn$	0.91 + 0.10	0.64 + 0.04
$Bu_4(2)Sn$	4.71 + 1.50	2.59 + 0.68
$Bu_4(1)Sn$	1.74 + 0.38	0.97 + 0.11

Bu = butyl, $CH_3CH_2CH_2CH_2$-
Bu(3) = but-3-enyl, CH_2=$CHCH_2CH_2$-
Bu(2) = but-2-enyl, CH_3CH=$CHCH_2$-
Bu(1) = but-1-enyl, CH_3CH_2CH=CH-
Pr = propyl, $CH_3CH_2CH_2$-
All = allyl, CH_2=$CHCH_2$-

Data for tri- and di-organotins are shown in Table 2. The two tributenyltin compounds are both more toxic than tributyltin and exhibit toxicities different from each other. Dibutenyltin is less toxic than dibutyltin. These compounds do show the expected trend of decreasing toxicity with double bond-induced increasing polarity.

TABLE 2

COMPARISON OF SATURATED AND UNSATURATED ORGANOTIN HALIDES

Compound	5 min EC50 (uM)	15 min EC50 (uM)
Bu_3SnCl	0.06 + 0.03	0.02 + 0.01
Bu_3SnBr	0.13 + 0.01	0.06 + 0.02
$Bu_3(3)SnBr$	0.44 + 0.05	0.27 + 0.03
$Bu_3(1)SnBr$	0.82 + 0.16	0.44 + 0.04
Bu_2SnCl_2	2.11 + 0.69	1.09 + 0.23
$Bu_2(3)SnBr_2$	5.13 + 0.69	2.82 + 0.22
Ph_3SnCl	0.36 + 0.08	0.14 + 0.02

Bu = butyl, $CH_3CH_2CH_2CH_2$-
Bu(3) = but-3-enyl, CH_2=$CHCH_2CH_2$-
Bu(1) = but-1-enyl, CH_3CH_2CH=CH-
Ph = phenyl

The reversal in toxicity response in the tetraorganotin and triorganotin compounds when unsaturation is introduced would seem to indicate that either steric or electronic effects may be at least as important as hydrophobicity as a predictor of toxicity. Although the possible effect of having a mixture of stereoisomers present in compounds with the double bond in the 1 or 2 position has not been taken into account, the variation in toxicity shown with placement of the double bond would seem to corroborate this observation. Hansch and Leo (19) noted that while highly specific biological response does show dependence on relative hydrophobicity, it is difficult to analyse because specificity arises out of the additional

crucial steric and electronic effects. Laughlin et al (20) found molecular size to be a better predictor of toxicity than simple fragment correlations. This approach might better explain the reversal in toxicity found in unsaturated organotin compounds.

CONCLUSIONS

EC50 data from bioluminescent bacteria bioassays correlates both qualitatively and quantitatively with most other bioassay procedures that have been used for organotin compounds. The method is rapid and relatively inexpensive. Data obtained on experimental compounds indicates that the method is valuable for assessing the relative toxicities of similar compounds.

ACKNOWLEDGMENTS

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